GM2 activator protein inhibits platelet activating factor signaling in rats

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Abstract

Platelet activating factor (PAF), an endogenous bioactive phospholipid, has been documented as a pivotal mediator in the inflammatory cascade underlying the pathogenesis of many diseases including necrotizing enterocolitis. Much effort has been directed towards finding an effective *in vivo* inhibitor of PAF signaling. Here, we report that a small, highly stable, lysosomal lipid transport protein, the GM2 activator protein (GM2AP) is able to inhibit the inflammatory processes otherwise initiated by PAF in a rat model of necrotizing enterocolitis. Based on behavioral observations, gross anatomical observations at necropsy, histopathology and immunocytochemistry, the administration of recombinant GM2AP inhibits the devastating gastrointestinal necrosis resulting from the injection of rats with LPS and PAF. Recombinant GM2AP treatment not only markedly decrease tissue destruction, but also helped to maintain tight junction integrity at the gastrointestinal level as judged by contiguous Zonula Occludens-1 staining of the epithelial layer lining the crypts.

Keywords

PAF; GM2AP; Inflammatory diseases; Necrotizing enterocolitis; Zonula Occludens-1; Lysosomal lipid binding protein

Introduction

Platelet activating factor, 1-*O*-alkyl-2-acetyl-sn-glycero-3-phosphocholine (PAF), an extremely potent bioactive phospholipid, is an endogenous mediator of platelet aggregation, inflammation, and anaphylaxis, which is released by a variety of cell types within the lesion [1]. Elevated levels of PAF further induce expression of other inflammatory factors including TNF- α and IL-1 β [2,3]. The production of PAF and the ensuing inflammatory response after injuries, such as trauma, haemorrhagic shock, sepsis, acute pancreatitis, and

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severe burns can lead to general organ dysfunction and failure, which has a mortality rate of >50% [4]. Inflammatory mediators, such as PAF, also play a key role in the pathogenesis of acute respiratory distress syndrome [4], and in necrotizing enterocolitis (NEC) [3,5]. In the latter case PAF, toll-like receptors and activation of the cytokine inflammatory cascade in concert play a pivotal role in the loss of an intact mucosal barrier, as well as in mucosal injury [6]. Additionally, PAF is known to induce severe endothelial barrier leakiness and the increased vascular permeability associated with inflammatory diseases. It has recently been reported that PAF-binding to its receptor induces the activation of Rho GTPase Rac1 by its guanine nucleotide exchange factor Tiam1, and a relocation of proteins Zonula Occludens-1 (ZO-1) and VE-cadherin from the inter-endothelial junctions, resulting in the formation of numerous gaps [7]. PAF has prolonged effects *in vivo* despite a very short plasma half-life suggesting the involvement of secondary mediators or, that a pool of PAF residing in the plasma membrane of the cells is released slowly over time [3]. PAF acts through specific receptors present on the membrane of responsive cells, *i.e.* neutrophils, resulting in a cascade of events that mediates the release of internal calcium stores [8]. PAF-acetyl hydrolase (PAF-AH) is a well characterized enzyme that can inactivate circulating forms of PAF [9]. However, in clinical trials recombinant PAF-AH (rPAF-AH) did not show sufficient efficacy in either human asthma or sepsis [10]. The reasons for this remain unclear, although it has been suggested that PAF-AH may have both a pro- and anti-inflammatory role, depending on the concentration and the availability of potential substrate [11]. Additionally PAF-AH can only utilize circulating PAF as a substrate and thus cannot reduce levels of PAF stored in the plasma membrane [9]. Recently, it has been discovered that another protein, the GM2 activator protein (GM2AP) can specifically bind and hydrolyze both soluble and membrane-bound forms of PAF *in vitro* [12,13].

GM2AP is a small (20 kDa), stable (heat stable at 60 °C), long-lived, protease-resistant protein that normally resides in the lysosome. This monomeric protein has been isolated and extensively characterized in our and other laboratories (reviewed in [14,15]). Its proven *in vivo* biological function is to act as a substrate specific cofactor for the lysosomal enzyme β -hexosaminidase A (Hex A) in its hydrolysis of GM2 ganglioside. The majority of GM2 ganglioside is produced through the breakdown of higher gangliosides, *e.g.* GM1 ganglioside, which are primarily found in neuronal cells. GM2AP is crucial for life, as patients who are genetically deficient usually die by the age of 5 with the AB-variant form of GM2 gangliosidosis, one of a family of three severe neurodegenerative diseases (reviewed in [14,16]). Recombinant GM2AP (rGM2AP) is able to inhibit PAF signaling in human neutrophils at a neutral pH and in the presence of cell medium containing a complex mixture of proteins [17]. In co-crystallization studies, PAF was bound within an accessible central hydrophobic cavity formed by a novel fold found in GM2AP. This fold is composed of eight-strands of anti-parallel β -pleated sheets whose shape resembles that of a cup, *i.e.* open at one end and closed at the other. Interestingly, PAF was actually reported to be hydrolyzed within this β -cup yielding inactive lyso-PAF [13]. Additionally, we [18] and others [19], have reported that all human cell types so far tested can endocytose extracellular GM2AP, transporting it to the lysosome by a mechanism that is independent of either the presence of its single Asn-linked carbohydrate moiety (present in GM2AP but not in rGM2AP) or whether or not it has formed a glycolipid complex. Thus rGM2AP has the potential to act in

a manner similar to a PAF-antibody, binding PAF and removing it from either the plasma membrane or the circulation leading to its final degradation by *in situ* hydrolysis or transfer to the lysosome.

In the present report we evaluate the ability of rGM2AP to inhibit *in vivo* the effects of exogenously administered PAF. For this purpose we utilized an accepted rat model of NEC induced by the injection of LPS and PAF [20].

Materials and methods

Animals

Adult male Sprague–Dawley rats (200–250 g of body weight) from Charles River Breeding Laboratory (Canada) were used in all experiments. Handling of the animals and experimental procedures were performed according the “Guide for the care and use of laboratory animals” (National Academic Press, Washington DC, 1996) and approved by the Animal Care Committee of the Hospital for Sick Children.

NEC induction

The rat model of ischemic bowel necrosis mimicking NEC was used as previously reported [20]. NEC induction was produced by an intravenous (i.v.) injection of LPS/PAF mixture (40 µg LPS, 2 µg PAF), designated as T0 for all the animal groups and experimental protocols. LPS/PAF was injected via the tail vein instead of the mesenteric artery to reduce stress and allow for the multiple injections required. Bacterial LPS from *Salmonella typhosa* and PAF were purchased from Sigma Aldrich (St. Louis, MI). Solutions for administration to animals were prepared in 0.9% saline before each experiment. The Laboratory Animal Services provided approved analgesic, and anesthetic.

rGM2AP treatment protocol

Rats were randomly distributed in groups of 10. All groups received analgesia and anesthesia; the first by subcutaneous (s.c.) injection 30 min prior to LPS/PAF; and the second by a mixture of anesthetic and oxygen/nitrous oxide. Human recombinant GM2AP (rGM2AP) was prepared as reported [12,21] sterile filtered and stored at 4 °C. Human rGM2AP was given either once (1 h before the PAF/LPS injection) or twice (1 h before and 1 h after) for treatment/prevention of NEC like-lesions at 1.2 mg/kg. At T-1 h, rGM2AP was given by tail i.v. and at T + 1 h, by s.c. injection. A mock treated group of animals received at T0 an i.v. injection of 0.9% saline; similarly they received saline injections instead of rGM2AP. After each injection the animals were returned to a single cage for subsequent injections, recovery and close monitoring. At T + 3 h, after the induction of acute NEC, animals were euthanized.

Behavioral observations

Several parameters were defined to monitor the overall behavior of the rats during the time frame of the experiments. They allow an estimation of the animals’ response to the different treatment given (Table 1). For the duration of the experimental protocol the animals were

closely observed for level of activity, signs of distress, and presence/aspect of feces in the cage as signs indicating morbidity and the onset and progression of acute GI inflammation.

Assessment of gross anatomy at necropsy and tissues/blood collection

After sacrifice, animals were immediately autopsied with a visual anatomical examination of the major organs (purple discoloration reflecting the hemorrhagic and/or obvious necrosis, flaccidity of the stomach, loss of normal luster) and tissues samples collected, including representative sections of the gastrointestinal tract (stomach, proximal and distal intestine, colon). Tissues and organs were rinsed and fixed for histological assessment and/or immunostaining.

Histopathology

Tissues were fixed in formalin, embedded in paraffin and 5 μ m cross-sections cut. Sections were mounted on glass slides, stained with hematoxylin and eosin (H&E), and assessed for histopathological changes. Observations within each group of rats and comparisons between groups in the same experiment were made. The overall aspect of the gut and intestine sections, and tissue abnormality was noted. Hyperplasia, tissue architecture, vasodilatation, and disruption of muscle layer were also noted and compared between the different groups of animals.

Immunocytochemical assessment of tight junction integrity of the GI epithelium

Integrity of the epithelial cell barrier in stomach and intestine was assessed by immunodetection of epithelial cell tight junctions with antibody to Zonula Occludens-1 (ZO-1), from Zymed Laboratories Inc. (San Francisco, CA). Briefly, paraffin sections of fixed GI tissues were deparaffinized and rehydrated. Antigen retrieval was performed; endogenous peroxidase and nonspecific binding were blocked prior to the application of rabbit anti-ZO-1. After several washes sections were incubated with the broad spectrum PolyHRP (Zymed) antibody, washed again and finally incubated with tyramine-Alexa 488, prepared as previously reported [22]. Final washes were performed and sections mounted in 50% glycerol in borate buffer. Sections were viewed in an Olympus epifluorescence microscope and images captured by Qcapture.

Results

Induction of NEC

As a first step in optimizing the rat NEC model a series of animal experiments were conducted to validate and determine the optimal dose of LPS/PAF for induction of NEC-like lesions, as well as the schedule of injections and dosage of rGM2AP. Basically, LPS/PAF dose and time of administration were varied to arrive at a sublethal schedule that would induce NEC acutely and allow animals to recover subsequently. Treated animals demonstrated signs of morbidity within 30 min as compared to saline injected controls, with some variation in the severity of the response (Table 1).

Whereas, mock treated animals displayed a normal anatomy (Fig. 1A), necrotic hemorrhagic lesions, similar to previously reported human NEC lesions [20], in both stomach and

intestinal mucosa were evident in all LPS/PAF treated animals at the gross anatomical level (Fig. 1B). At the histopathological level LPS/PAF treated animals showed an obvious hemorrhagic appearance in both intestine and stomach with extensive tissue destruction distal to the crypts which appeared to be somewhat spared (Fig. 2B and E). Intestinal luminal contents contained extensive sloughings of the villi.

To assess the integrity of the GI epithelium we immunostained for ZO-1, a tight junction protein essential for maintaining the epithelial cell barrier and whose relocation from inter-endothelial junctions is a direct result of PAF-signaling [7]. We found that, as expected, untreated animals showed a contiguous tight junction barrier approximating the villous tips (Fig. 3A and D). In stark contrast LPS/PAF treatment completely disrupted epithelial cell integrity along the villi and left tight junction integrity relatively intact but disorganized in the crypts (Fig. 3B and E).

rGM2AP treatment blocks the pathogenic process of LPS/PAF induced NEC

Opposite to that observed in the GI-tract of LPS/PAF treated rats, in animals given rGM2AP at 1 h pre and post LPS/PAF treatment, both stomach and intestinal tissues grossly exhibited anatomical features resembling mock treated animals (compare Fig. 1C to Fig. 1A). A significantly lesser degree of tissue destruction, cell sloughing, and a low level of hemorrhage (mainly vasodilatation of capillaries residing within the interstitium of the villi) was evident histologically (Fig. 2C and F). Furthermore, the animals' intestinal and stomach epithelia retained a large degree of their epithelial cell-cell adhesion, as shown by ZO-1 staining (Fig. 3C and F), and the integrity of their epithelial barriers were well maintained. These data correlate with the normalization observed in their behavior and functions (Table 1). In short, tissue destruction was significantly minimized as well as vasodilatation with no indication of hemorrhage. Human rGM2AP itself did not produce adverse effects when control animals received an equivalent dose of rGM2AP or when LPS/PAF treated animals received multiple doses of rGM2AP over 5 days (data not shown).

Discussion

It has been shown that the ileum has the highest expression of PAF receptor, although it is also relatively abundant in other intestinal regions [3]. In our rat model the intestine was significantly more affected than the stomach and rGM2AP treatment was accordingly more effective in stomach. The current literature indicates that circulating PAF is removed from inflammatory sites by PAF-acetyl hydrolase (PAF-AH). However, although PAF is clearly involved in bronchial asthma, neither the administration of rPAF-AH [23] or currently used PAF-receptor antagonists [24] have proven to be beneficial against asthmatic crisis. Treatment with PAF-receptor antagonists may be complicated by the ability of PAF in the plasma membranes of one cell to interact with the PAF-receptor in the plasma membrane of another cell in close proximity. Thus, patches of bound PAF could present receptors on adjacent cells with a much higher localized concentration of PAF, as compared to circulating PAF levels. This would result in a decreased apparent K_d for the receptor-PAF complex causing receptor-antagonist to lose their effectiveness in blocking PAF signaling. Similarly membrane bound PAF is also not a substrate for PAF-AH, which is only able to hydrolyze

the circulating forms of PAF [9]. We suggest that targeting membrane-bound pools, as well as circulating forms of PAF with rGM2AP may prove more effective.

rGM2AP appears to be a fast-acting PAF-antagonist, as it was able to mostly inactivate 2 µg of PAF injected into the circulation of the rats. Clearing and/or inactivation of PAF abrogates many of the subsequent downstream events that lead to tissue destruction. Vascular delivery of endogenous GM2AP to sites of inflammation does not appear to occur under homeostatic conditions, because of its low level of expression and its lysosomal localization. However, by supplying rGM2AP exogenously we show that the NEC-like pathogenic process induced by the injection of LPS and PAF is inhibited and GI tissue destruction significantly minimized to a low-grade inflammation with subsequent resolution. Furthermore, residual rGM2AP would not be deleterious since many cell types can eventually endocytose rGM2AP to relocate it back to lysosomes [18]. The lack of an *N*-linked carbohydrate on the rGM2AP could also be an advantage, as it would likely increase its half-life in the circulation. Finally, since proteolysis is dominant during tissue destruction, the resistance of rGM2AP to proteases would preserve its activity in affected sites. This is a novel example of how changing the levels and cellular localization of a protein can result in it expressing a heretofore-unexpected biological activity.

For the following reasons there should be little problem in translating the 1.2 mg/kg dose of rGM2AP given to the rats in this report to the treatment of humans. Firstly, the production of large amounts of functional rGM2AP is possible in transformed bacteria after re-folding from inclusion bodies (~100 mg/L culture) [12]. Secondly, lysosomal proteins are generally well tolerated by patients, even when given in large amounts over long periods of time. For example, there are presently at least 6 lysosomal storage diseases for which enzyme replacement therapy has either been approved or is in a clinical trial. In these cases the deficient enzyme is given to patients in monthly doses ranging from 0.4–160 mg/kg for the remainder of their lives. The most commonly used of these therapies is the administration of recombinant acid β-glucosidase to patients with type I Gaucher disease. This treatment was approved for use in 1991 at a monthly dose of 3.2 mg/kg (reviewed in [25]). Thus, rGM2AP is a good candidate for introduction as a frontline therapeutic intervention in acute diseases, such as NEC, where PAF is a critical inducer of the inflammatory response, which plays a key role in their pathogenesis.

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Fig. 1.

Gross anatomical features of the gastrointestinal (GI) tract at necropsy. Note the hemorrhagic aspect of the GI in (B), blood vessels on the surface of the stomach, intestine and cecum are engorged and very prominent (arrows), a strong purple discoloration of the lower stomach and some loops of the intestine (arrowheads) are present. In contrast (A) and (C) show an essentially similar normal overall pinkish color of the GI tract with few visible blood vessels. L, liver; S, stomach; I, intestine; C, cecum.

Fig. 2.

Histopathology of the stomach and intestine. These H&E stained sections show the effect of rGM2AP administration on a rat model of acute NEC. Typical pathological NEC-like changes were observed in both stomach and intestine with severe alterations in tissue integrity occurring focally along the GI tract. In the stomach (A, B, and C) extensive hemorrhage (arrow in B) and necrotic changes to mucosal tissue were found, including edema and infiltration with inflammatory cells in (B). The normal villous crypt architecture (A) disappeared, except for remnants of basal crypts areas (B arrowhead). The stomach wall appeared not to be breached. Treatment with rGM2AP essentially blocked the major inflammatory and necrotizing tissue alterations, except for a small amount of epithelial sloughing with minimal edematous changes (C). In the intestine (D, E, and F) the normal villous structure (D) were severely disrupted (E) with evidence of hemorrhage (arrow), edema and inflammatory infiltrate giving an appearance of a granulomatous-like wound lesion (Gr) with extensive sloughing of the epithelium (asterisks); only remnant basal crypt areas could be seen (E arrowheads). Intestinal wall architecture was not affected under these conditions. As in the stomach, administration of rGM2AP blocked most of the severe NEC-like pathological changes (F). Only a small degree of vasodilatation, epithelial cell sloughing and disruption of villous organization occurred during treatment. The difference in pathological changes (B and E versus C and F) correlated with morbidity (see Table 1).

Fig. 3.

Effect of rGM2AP administration on the integrity of ZO-1 positive (white arrows) epithelial tight junctions in experimental NEC in rats. (A) Mock treated rat stomach showing contiguous ZO-1 staining of the epithelial layer lining the crypts except at the luminal apices where normal sloughing occurs; (B) NEC induced stomach epithelium showed severe loss of ZO-1 staining indicating tissue destruction, except at the base of the crypts (damaged cells show non-specific immunofluorescence); (C) in rGM2AP treated stomach epithelium ZO-1 staining was retained along the major aspects of most crypts, indicating only minor tissue destruction. In the intestine (3D, E, and F) ZO-1 staining patterns were comparable and match the histopathological changes visualized by H&E staining (Fig. 2). However, ZO-1 staining better defined the degree of functional loss and the general sparing of basal crypt and villous areas in LPS/PAF-induced NEC.

Table 1

Behavioral observations.

Parameters	Animal groups		
	Mock treated ^a	NEC induced ^b	rGM2AP ^c
Behavior	Animal mobile, curious and grooming	Unresponsive within 15–30 min ^d	Animal more active than NEC, but less than Mock
Appearance	Normal	Hunched over within 15 min, fur ruffled within 20 min	Normal for ~75% of rats
Locomotion	Normal	Not moving	Normal for ~75% of rats
Respiration	Normal	Fast and shallow	Normal for ~75% of rats
Defecation	Restarted within 3 h	None within 3 h, ^e Restarted within 8–12 h (some bloody)	Restarted within 3 h
Eyes	Open	Closed	Open

^aControl group, mock treated animals receiving only saline injections.

^bRats receiving LPS (40 µg) plus PAF (2 µg) at T0 to induce NEC like lesions.

^cRats receiving LPS plus PAF injection and treated with rGM2AP.

^dTime indicated is relative to T0.

^eData obtained from additional experiments with extended observation time frame (not shown).